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Development of Low Cost Peptide-Based Anti-Hepatitis C Virus Screening and Confirmatory Assays: Comparison With Commercially Available Tests

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Screening and confirmatory low cost reagent tests have been developed for detection of anti-hepatitis C virus (HCV). Assays are based on the use of specific synthetic peptides from several structural and non-structural viral proteins. The efficacy of the screening anti-HCV EIA-Spep assay was compared with both Abbott EIA 2.0 (Abbott Laboratories, North Chicago, IL) and Ortho EIA 2.0 (Ortho Diagnostic Systems, Raritan, NJ) anti-HCV detection kits and the confirmatory EIA-Cpep assay was compared with the Abbott Matrix anti-HCV confirmation test. In the EIA-Spep, a pool of 3 peptides was added to each well of a microtiter plate. In EIA-Cpep, each well was separately coated with 1 of 4 peptides and 1 recombinant protein. A total of 867 blood donor samples from Costa Rica tested simultaneously with the 3 screening assays yielded the same specificity and negative predictive values of ≥99.9% and 100%, respectively. A comparative study on voluntary blood donor samples from Honduras, Nicaragua, and El Salvador using the 2 anti-HCV confirmatory assays revealed different patterns that are 46% positive, 24% indeterminate, and 30% negative with the EIA-Cpep assay vs. 31% positive, 48% indeterminate, and 21% negative with the Matrix assay. A study of 71 patient samples from Costa Rica showed a higher correlation between initially reactive samples when analyzed by the Abbott and Ortho kits, than when the assay results were compared between the Abbott and EIA-Spep kits; the latter detected 7 and 15 non-reactive samples, respectively. These results could reflect the use of a similar antigen source for the 2 commercial assays. The presence of HCV RNA in a group of 29 samples analyzed was related to the simultaneous reactivity in all 3 screening assays. None of the discordant samples had detectable levels of HCV RNA. Economic difficulties for health care services in the developing countries of Central America have prevented implementation of routine anti-HCV blood donor screening tests. This is likely to be the primary reason for uncontrolled dissemination of HCV, and the lack of identification of potential high risk groups. Alternative low cost reagents developed locally as described in this article could be a useful tool in the control of HCV spread throughout the developing world. ***J. Med. Virol. 58:221–226, 1999.***

KEY WORDS: anti-HCV; synthetic peptides; screening confirmation; blood donors

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease throughout the world, with an estimated 200 million infected individuals [Dhillon and Dusheiko, 1995; NIH, 1997]. The transmission route is predominantly parenteral, although in >30% of chronic cases the infection route is not established [Alter, 1994]. Thus far, the vast majority of infections have been through blood transfusion [Garson et al., 1990; Aach et al.]. Therefore, priority for control should be given to routine screening for HCV of all blood-related products. The prevalence rate of HCV-infected blood donors in different geographic areas fluctuates from <0.1% to >5%, and the risk of HCV infection after receiving 1 non-screened blood unit varies between 1% and >15% [Alter and Seeff, 1993; Esteban, 1995].

The determination of specific antibodies to HCV proteins is used as a marker for the diagnosis of HCV infection. Several commercial screening assays available for anti-HCV are based on the use of recombinant

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proteins to detect the presence of antibodies directed against a pool of different epitopes of the virus [Alter et al., 1989; Kuo et al., 1989]. Many screening tests lack specificity and <50% of initial antibody reactive samples from blood donor populations are positive when retested in a confirmatory setting [Tibbs et al., 1991; Allain et al., 1992; García et al., 1996]. In confirmatory assays a specific antibody pattern against different HCV epitopes is determined and most of these are based on the use of recombinant proteins as antigen source [Chaudhary and Jacobsen, 1994]. However, the use of synthetic peptides as antigen for specific HCV epitopes has also been shown to be an adequate alternative for both screening and confirmatory assays [Kotwal et al., 1992; Ishida et al., 1993; Khudyakov et al., 1995]

In developing countries, there is a lack of implementation of routine testing procedures for the detection of HCV-infected blood units due to the high cost burden of commercial reagents. Therefore, there is a need to develop alternative reagents [PAHO, 1997].

The aim of this study was to develop low cost in-house screening and confirmatory assays using synthetic peptides as the primary antigen source and to compare these assays with commercial reagents currently in use.

MATERIALS AND METHODS

Study Populations

Three different groups of blood donors and one group of patient samples were used. The first group was used in order to evaluate the specificity of the "in-house" International Center for Medical Research and Training (ICMRT) EIA-Spep (S-screening) assay for anti-HCV. This population of 867 blood donor samples from Costa Rica was simultaneously tested with the ICMRT EIA-Spep assay and 2 anti-HCV commercial assays: Abbott EIA 2.0 (Abbott Laboratories, North Chicago, IL) and Ortho EIA 2.0 (Ortho Diagnostic Systems, Raritan, NJ) kits. A comparative analysis was performed using results from the 3 screening assays.

Evaluation of the "in-house" ICMRT EIA-Cpep (C-confirmatory) kit was done using a second group of 71 initially reactive voluntary blood donor samples by the Abbott EIA 2.0 screening assay from Honduras, Nicaragua, and El Salvador, collected during 1994–95 [García et al., 1996]. These samples were retested using the Abbott Matrix confirmation test. A comparative study was done between the 2 assays analyzing confirmation criteria (positive, indeterminate, negative) and reactivity to specific epitopes of each assay in discordant samples.

The third study group, from Costa Rica, comprised 71 initially reactive patient samples by the anti-HCV Abbott EIA 2.0 screening kit, and were referred to us from the health system for diagnosis of acute or chronic hepatitis. They were retested with the Ortho EIA and the ICMRT EIA-Spep screening tests. Confirmatory analyses were done using the EIA-Cpep reagent kit. A comparative analysis among the 3 screening assays was performed and the distribution of reactivity to specific epitopes in the EIA-Cpep assay was tabulated.

A companion study was done using samples from the 2 latter groups above (71 blood donors and 71 patients), evaluating the reactivity in the confirmatory setting to each HCV epitope.

Finally, the last study group included 29 initially anti-HCV reactive samples by Abbott EIA 2.0 from Nicaraguan voluntary blood donors collected during 1996–97. These were retested by the Ortho EIA 2.0, ICMRT EIA-Spep, and EIA-Cpep kits and analyzed for HCV RNA by polymerase chain reaction (PCR) followed by hybridization. These results were compared with the pattern obtained in all 3 screening assays and the ICMRT EIA-Cpep test.

Peptide Synthesis and Labeling

Peptides were synthesized on a PerSeptive Biosystems (Framingham, MA) 9050+ Peptide Synthesizer using FMOC chemistry. The peptides were cleaved from resin with 5% phenol in trifluoroacetic acid according to the manufacturer's instructions. All synthetic peptides were synthesized with an additional cysteine at the NH_2-terminal. Purification was performed by reverse phase-high performance liquid chromatography (RP-HPLC). RP-HPLC was also used to verify purity. Dissolved peptides in 0.15 M phosphate-buffered saline (PBS) and 1 mM EDTA were labeled with ImmunoPure Biotin-HDPD (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Labeled product was applied to a Sephadex G-10 column equilibrated with 0.15 M PBS and 1 mM EDTA, and then eluted with the sample solution.

Screening Assays

Commercial sources. For anti-HCV detection, 2 commercial and 1 in-house methods were used. The commercial assays were EIA 2.0 screening kit from Abbott Laboratories and EIA 2.0 kit from Ortho Diagnostic Systems. The Manufacturer's instructions were followed in both methods.

In-house ICMRT EIA-Spep assay. HCV peptides chosen for synthesis were selected based on their highly reactive epitopes (Table I). Anti-HCV detection EIA was performed on 96-well Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) precoated with 0.1 μg/well of streptavidin (Sigma Chemical Co., St. Louis, MO) dissolved in 0.15 M PBS (pH 7.4) at 4°C for 48 hr and coated with 30 pg of equal amounts of the first 3 synthetic peptides (pepC-1, pepC-2, and pepNS4) pooled in PBS buffer, by overnight incubation at room temperature. Postcoating was done with a solution of 2% polyvinyl pyrrolidone (PVP-40) (Sigma Chemical Co.) in 0.01 M $NaHCO_3$ buffer (pH 9.6) at room temperature for 4 hr. Serum specimens were diluted 1:40 (Tris base 0.5 M, NaCl 0.05 M, 2.5% dried skim milk, and 0.1% bovine serum albumin, pH 7.4) and added to microtiter wells, prior to incubation at 40°C for 20 min. After washing 5 times with PBS containing 0.5% Tween-20, plates were incubated with

TABLE I. Sequence of HCV Peptides

Peptide name	Amino acid (AA) sequence	AA number	Length[a]	Reference
pepC-1	CKPQRKTKRNTNRRPQY	5–20	16	[Kotwal et al. 1992]
pepC-2	CDVKFPGGQIVGGVYLLPRRGPRLG	21–44	24	[Ishida et al, 1993]
pepNS4	CAFASRGNHVSPTHYVPESDA	1921–1940	20	[Khudyakov et al, 1995]
pepNS5	CPPLLESWKDPDYVPPVVHG	2295–2313	19	[Khudyakov et al., 1995]

[a]AA numbers do not include the N-terminal cysteine.

goat anti-human IgG labeled with peroxidase (Sigma Chemical Co.) at 40°C for 20 min and again washed 5 times. The reaction was developed with TMB and OD was measured at 450 nm (Sigma Chemical Co.). The criterion for reactivity was taken as ≥3.0 standard deviation (SD) added to the mean value of previously determined negative controls (NC). A test was considered valid when the positive/negative ratio was ≥10-fold.

Confirmatory Assays

Abbott Matrix assay. This test is based on detection of specific antibodies reacting with several recombinant proteins of HCV: core, NS3, and NS4. NS4 is included as 2 separate antigens, NS4(y) and NS4(e).

ICMRT EIA-Cpep. Specific antibodies were detected against an in-house kit containing 4 synthetic peptides—pepC-1, pepC-2, pepNS4, and pepNS5 (Table I)—and a recombinant NS3 (rNS3) protein (BINEM, Beijing, China). For the peptides, plates were prepared in the same manner as described above with EIA-Spep, except that each peptide was added separately to a different well. The NS3 protein dissolved in 0.01 M $NaHCO_3$ buffer (pH 9.6) was also added to different wells at a concentration described by the manufacturer. Serological assays were carried out in the same manner as with EIA-Spep. The criteria used to determine reactivity of the ICMRT EIA-Cpep kit were as follows. Samples were considered reactive if readings were ≥3 SD added to the mean OD 450 nm value of negative controls. Samples were considered positive if they reacted against >1 antigen. Samples reacting against 1 antigen or that had reactivity against all peptides within 1 cutoff value range were considered indeterminate. If a sample was not reactive against any of the antigens, it was considered to be negative.

RT-PCR for HCV RNA

The Quanti-Path (CPG, Inc., NJ) kit was used according to the manufacturer's recommendations as follows: RNA was extracted from 140 μl of plasma using viral RNA kit (Qiagen Co., Chatsworth, CA) spin columns. RNA was reverse transcribed in a 40 μl reaction containing 100 pmol of anti-sense primer from the 5′NTR of HCV, 2.5 mM $MgCl_2$, 1 mmol of each deoxyribonucleotide triphosphate (dNTP), 0.6 mM DDT, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 U RNase inhibitor (Promega Corp., Madison, WI), and 50 U Super Script II reverse transcriptase (GIBCO BRL, Grand Island, NY). The mixture was incubated at 95°C for 5 min and at 42°C for 45 min. PCRs were performed using the total cDNA volume added to 40 μl of PCR mixture [100 pmol of sense primer, 2.5 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.3), 50 mM KCl, and 5 U Taq DNA polymerase (Promega Corp.)]. The mixture was amplified for 40 cycles (95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec) in a Perkin Elmer (Oak Brook, IL) 2400 thermocycler. The amplified product was detected using the Quanti-Path plate hybridization system.

RESULTS

Initially, the specificity of the ICMRT EIA-Spep assay was analyzed together with 2 commercial kits in 867 Costa Rican blood donor samples (Table II). All assays revealed ≥99.9% specificity and a 100% negative predictive value.

Subsequently, comparison of the ICMRT in-house confirmatory assay EIA-Cpep was made vs. the commercial Abbott Matrix test, using 71 positively screened voluntary blood donors from Central America. Table III shows 46% and 31% positive confirmation, respectively. The percentages of negative samples were found to be 30% for the EIA-Cpep and 21% for the Matrix test, while indeterminate samples appeared to be 2-fold higher for the Matrix kit. These results indicate that there is a discrepancy in confirmation between the 2 assays.

It was noted that there were 14 discordant samples between indeterminate and negative samples. Of these, 10 were classified as negative by EIA-Cpep and indeterminate by Matrix, while 4 had the opposite pattern. The detailed description of which epitopes exhibited specific reactivity for the 14 samples is shown in Table IV. A predominant reactivity to NS4 was observed for the negative EIA-Cpep and indeterminate Matrix samples, while the negative Matrix samples resulted in a more diverse reactivity pattern.

In a different study, results obtained with 71 Costa Rican patient samples initially reactive with the Abbott EIA 2.0 assay were retested with the Ortho 2.0 kit and then compared with the EIA-Spep and EIA-Cpep tests (Table V). A higher correlation of initially reactive samples was found between Abbott and Ortho than between Abbott and EIA-Spep, detecting 7 and 15 nonreactive samples, respectively. None of the 15 EIA-Spep negative samples was positive in the EIA-Cpep confirmation assay; 1 was indeterminate and 14 were negative. Of the 7 Ortho EIA 2.0 negative samples, 1 was indeterminate and 6 were negative.

The distribution of reactivity to specific antigens in the EIA-Cpep kit from Central American blood donors as well as Costa Rican positive and indeterminate populations was analyzed as shown in Table VI. NS3

TABLE II. Evaluation of Specificity and Negative Predictive Value of ICMRT EIA-Spep, Ortho EIA 2.0, and Abbott EIA 2.0 Screening Kits in 867 Blood Donor Samples From Costa Rica

Screening assay	Reactive samples	Specificity (%)	Negative predictive value (%)
EIA-Spep	3	99.9	100.0
Ortho EIA 2.0	3	99.9	100.0
Abbott EIA 2.0	2[a]	100.0	100.0

[a]Two samples were reactive in all screening assays and confirmed positive by EIA-Cpep.

TABLE III. Comparison Between Confirmatory Assay Patterns of Abbott Matrix and ICMRT EIA-Cpep Kits, Using a Group of Blood Donor Samples From Central America

	Matrix			Total
EIA-Cpep	Positive	Indeterminate	Negative	(%)
Positive	22	11	0	33 (46)
Indeterminate	0	13	4	17 (24)
Negative	0	10	11	21 (30)
Total (%)	22 (31)	34 (48)	15 (21)	71 (100)

apparently discriminates between positive and indeterminate results better than the other antigens, since only 1 sample from an indeterminate blood donor was reactive to NS3. However, NS3 was not as sensitive as pepC (1 and 2) in the positive population.

The final evaluation, shown in Table VII, reveals the correlation between results of the 3 screening assays, the EIA-Cpep confirmatory test, and the results of HCV RNA detected by PCR and hybridization. Of the 29 samples analyzed, 23 were reactive by all antibody assays and 20 had detectable HCV RNA, while none of the samples with antibody discordant results had detectable levels of HCV RNA.

DISCUSSION

The first available anti-HCV screening assays developed by the Chiron Corporation were based on the detection of antibodies against the non-structural protein NS4 [Alter et al., 1989; Kuo et al., 1989]. However, these tests lacked high sensitivity, and later generation kits were developed for the simultaneous determination of antibodies against additional structural and non-structural HCV proteins [Kleinman et al., 1992].

Core proteins have been shown to contain the most immunogenic regions, followed in sensitivity by the non-structural NS3, NS4, and NS5 proteins [Katayama et al., 1992; Vernelen et al., 1994]. The two *env* regions are highly variable and therefore less useful as diagnostic tools [Barrera et al., 1996; Cerino et al., 1997].

Both recombinant proteins and synthetic peptides have been shown to be adequate antigen sources, excluding the NS3 region, where no synthetic peptides have yet been used successfully for antibody detection [Claeys et al., 1995]. In this study, we have shown that a combination of peptides including 2 core, 1 NS4 and 1 NS5, as well as an NS3 antigen can be used successfully in screening and confirmatory assays for detection of specific antibodies to HCV in blood donor and patient populations. It has been shown to have a comparable specificity and sensitivity to commercially available reagents. In the "in-house" confirmatory assay (EIA-Cpep), a better resolution of positive vs. indeterminate samples was observed than with the Abbott Matrix assay. These findings correlate well with earlier studies comparing 3 commercial third-generation confirmatory assays, in which the Abbott Matrix kit revealed the highest percentage of indeterminate results [Chaudhary and Jacobsen, 1994; Dussaix et al., 1994].

Further, comparing 3 screening assays of patient samples (Table V), a higher correlation was found between Abbott and Ortho initially reactive samples (64/71) than both had with the EIA-Spep kit (56/71). However, none of the discordant samples confirmed positive by the ICMRT-Cpep kit, suggesting that Abbott and Ortho share a similar false positive sample population. This is a possibility since both assays use similar recombinant antigens and differ from the EIA-Spep kit, which is based upon synthetic peptide antigens. In support of this hypothesis, confirmatory assays reveal that the discordance between the ICMRT in-house system and the commercial Abbott test kit was related mostly to reactivity against specific NS4 without reactivity to another HCV epitope. This event can often be caused by non-specific antibody cross-reactivity, in the absence of detectable HCV RNA [Bresters et al., 1993; Dow et al., 1996].

The use of HCV RNA as a confirmatory assay has been widely discussed and recently discounted by an HCV Expert Council Study Group [NIH, 1997]. However, PCR is still a useful tool to consider for evaluation of reactivity to specific epitopes of indeterminate samples [Marin et al., 1994; Lok and Gunaratnam, 1997].

The lack of routine screening of anti-HCV in blood safety programs in developing countries is probably a major cause of increasing HCV incidence. Most likely, the primary reason for not controlling such blood transmission is the cost burden of currently available HCV screening reagents. Presently, the unit price for anti-HCV test kits is 4–8 times higher than anti-HIV kits in Central America. Recalling the economic difficulties in developing countries to implement anti-HIV blood donor screening, an even more difficult task is faced in the control of HCV post-transfusion acquired infection. Also, there is lack of adequate epidemiological surveillance of HCV in this region regarding high risk groups and the general population.

Several international organizations (WHO, PAHO, IOAE) are trying to promote technology transfer and development, including use of available HCV diagnostic test reagents to help solve local health-related problems. However, there is a need to simultaneously make available adequate control materials and systems for evaluation and validation of locally developed HCV re-

TABLE IV. Specific Reacting Antigens in Confirmatory Assay Discordant Samples Observed for the Central American Blood Donor Population

Sample ID	Confirmation result		Reacting antigen	Ratio OD/cutoff
	EIA-Cpep	Matrix		
2638	Indeterminate	Negative	pepC-2	1.1
590	Indeterminate	Negative	pepC-1	1.5
2804	Indeterminate	Negative	pepNS4	1.1
2970	Indeterminate	Negative	All peptides	1.0–1.7[a]
560	Negative	Indeterminate	Core	7.3
2244	Negative	Indeterminate	NS4(y)	9.3
2971	Negative	Indeterminate	NS4(e)	1.7
576	Negative	Indeterminate	NS4(y)	1.4
3033	Negative	Indeterminate	NS4(y)	2.1
2940	Negative	Indeterminate	NS4(y)	6.5
1902	Negative	Indeterminate	NS4(y)	11.2
1900	Negative	Indeterminate	Core	2.5
2967	Negative	Indeterminate	Core	2.4
566	Negative	Indeterminate	NS4(y)	4.3

[a]Possible high background reactivity.

TABLE V. Anti-HCV Screening and Confirmation Results of 71 Costa Rican Patients

Screening result		Screening result using EIA-Spep								
			EIA-Cpep confirmation			Non-	EIA-Cpep confirmation			Total
Abbott 2.0	Ortho 2.0	Reactive	Positive	Indeterminate	Negative	reactive	Positive	Indeterminate	Negative	samples
+	–	2	0	1	1	5	0	0	5	7
+	+	54	43	10	1	10	0	1	9	64
Total		56	43	11	2	15	0	1	14	71

TABLE VI. Seroreactivity to Antigens Used in the Confirmatory EIA-Cpep Assay

Population	Total	EIA-Cpep result	Seroreactivity				
			pepC-1	pepC-2	pepNS4	pepNS5	rNS3
Blood donors[a]	33	Positive	33	33	31	17	17
	17	Indeterminate	13	9	5	5	1
Patients[b]	43	Positive	40	43	39	24	38
	12	Indeterminate	3	7	4	1	0

[a]Samples from the 71 Central American blood donors.
[b]Samples from the 71 Costa Rican patients.

TABLE VII. Comparison Between Anti-HCV Screening and Confirmatory Assays and HCV RNA

Screening result				EIA-Cpep confirmation				EIA-Cpep confirmation			
Abbott 2.0	Ortho 2.0	EIA-Spep	RT-PCR positive	Positive	Indeterminate	Negative	RT-PCR negative	Positive	Indeterminate	Negative	Total samples
+	–	–	0	0	0	0	4	0	0	4	4
+	+	–	0	0	0	0	1	0	0	1	1
+	–	+	0	0	0	0	1	1	0	0	1
+	+	+	20	19	1	0	3	3	0	0	23
Total			20	19	1	0	0	4	0	5	29

agents for its successful implementation. Only in this way can the technological gap between developed and developing countries be narrowed.

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